

Comparison of Antigenic Sites of Subtype-specific Respiratory Syncytial Virus Attachment Proteins

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SUMMARY

A panel of 19 monoclonal antibodies (MAbs) were used to probe the antigenic relationships between the G (attachment) proteins of A and B respiratory syncytial virus (RSV) subtypes (G_A and G_B). At least three and two antigenic sites were present on G_A and G_B , respectively, including a shared neutralizing site. Most of the antibodies had some degree of complement-independent neutralizing capacity, but in common was a large neutralization-resistant fraction of virus (range 13 to 78%). Passive administration of MAbs to the cross-reactive antigenic site reduced pulmonary virus titres of both A and B subtype virus in the cotton rat model. Protection with subtype-specific MAbs, however, did not always correlate with *in vitro* neutralizing capacity. The cross-reactive antigenic site appears to be stable to denaturation by polyacrylamide gel electrophoresis and is present on the unglycosylated and partially glycosylated forms of G_A and G_B by Western blot analysis of infected cell lysates.

INTRODUCTION

Respiratory syncytial virus (RSV), a negative-strand RNA virus of the paramyxovirus family, is the leading cause of severe lower respiratory tract infection in infants (Kim *et al.*, 1973). The viral genome encodes 10 proteins including two envelope glycoproteins which subserve attachment and penetration functions (Huang *et al.*, 1985). The attachment protein (G) has a protein backbone of 298 amino acids with an M_r of 32K but migrates by PAGE with an M_r of 84K to 90K because of extensive O-linked and N-linked glycosylation (Wertz *et al.*, 1985; Levine *et al.*, 1987). Viral penetration into cells and cell-to-cell spread of virus is mediated by the 70K fusion protein (F), a typical paramyxovirus fusion protein composed of disulphide-linked fragments, F_1 (48K) and F_2 (23K) (Walsh & Hruska, 1983). Neutralizing epitopes are present on both F and G proteins and, thus, are candidates for use in a subunit RSV vaccine (Walsh & Hruska, 1983; Walsh *et al.*, 1984). Immunization of cotton rats with purified F or G protein confers protection from challenge with RSV (Walsh *et al.*, 1987a).

Recently, two antigenically distinct subtypes of RSV, designated A and B, have been described independently from several laboratories (Anderson *et al.*, 1985; Mufson *et al.*, 1985; Storch & Park, 1987). Both antigenic and structural differences between subtypes have been described for several RSV proteins, including G and F (Ward *et al.*, 1984; Norrby *et al.*, 1986; Walsh *et al.*, 1987b; Morgan *et al.*, 1987). The most significant antigenic difference between A and B subtypes, however, resides on the G protein where amino acid homology is only 53% and antigenic relatedness is 5% (Walsh *et al.*, 1987b; Johnson *et al.*, 1987a). Mufson *et al.* (1985) reported that only one of six monoclonal antibody (MAb)-defined epitopes on G was present on RSV isolates of both subtypes. Additionally, convalescent human antisera and rabbit polyclonal

antisera to affinity-purified G from either subtype has little cross-reactivity, and neutralization is essentially subtype-specific (Walsh *et al.*, 1987*b*; Johnson *et al.*, 1987*b*; Hendry *et al.*, 1988). Consistent with these findings, immunization of cotton rats with a vaccinia virus vector expressing A subtype G (G_A) provided primarily subtype specific protection (Stott *et al.*, 1987; Johnson *et al.*, 1987*b*). In contrast, the F protein has a high degree of structural and antigenic homology between subtypes (Johnson & Collins, 1988). Thus it has been suggested that an optimal subunit RSV vaccine should contain G proteins representing both RSV subtypes and a single F protein.

In order to explore further the antigenic relationship and identify neutralizing epitopes on the G proteins, we analysed purified G protein from A and B subtypes (G_A and G_B) with a panel of MAbs. The results have identified a single neutralizing protective site, common to the G protein of both A and B subtypes.

METHODS

Virus and cells. HEP-2 cells were maintained in minimal essential medium (MEM) supplemented with 5% foetal calf serum (FCS), 1 mM-glutamine, penicillin and streptomycin. The Long and 18357 strains of RSV, laboratory prototypes of A and B subtypes respectively, were grown in HEP-2 cells and frozen at -70°C until use.

Purification of G proteins. The G proteins from Long and 18357 virus, designated G_A and G_B respectively, were purified by immunoaffinity chromatography as previously reported (Walsh *et al.*, 1984, 1987*b*).

Production of MAbs. Eleven MAbs were produced by two intraperitoneal immunizations of BALB/c mice with 10 μg of purified G_B with Freund's adjuvant, followed by fusion of spleen cells with X63/Ag8.653 myeloma cells as previously described (Walsh & Hruska, 1983). These MAbs are designated with the prefix C. In a similar fashion, six MAbs to G_A were produced and are designated with the prefix K. Two previously described MAbs (L7 and L9), produced by intranasal immunization with live Long RSV, were also used (Walsh & Hruska, 1983). All hybridomas were screened by indirect immunofluorescent assay (IFA) on infected HEP-2 cells (utilizing the homologous strain) and further characterized by enzyme immunoassay (EIA) and Western blotting against RSV proteins. Immunoglobulin type and subtype were identified by Ouchterlony double diffusion with mouse subtype-specific antisera (Meloy and Litton Bionetics). The concentration of MAb in ascites was determined by electrophoresis on cellulose acetate membranes, followed by scanning densitometry.

Virus neutralization. Neutralization of Long and 18357 RSV was performed by a plaque reduction assay. Briefly, 50 to 100 p.f.u. of virus were mixed with MAb (ascites for all MAbs except K5 and K9 in which ammonium sulphate-precipitated cell culture supernatant was used) for 30 min and then plaqued on monolayers of HEP-2 cells in 24-well plates. Neutralization was recorded as percentage plaque reduction compared to a control MAb (anti-17D yellow fever virus E protein). Complement was not added to the antibody-virus mixture.

PAGE and Western blots. PAGE and Western blots were performed as previously described using infected HEP-2 cell lysates as the antigen (Walsh *et al.*, 1984).

Competitive binding assay (CBA). MAbs were ammonium sulphate-precipitated from mouse ascites and labelled with biotin according to a published method (Wagener *et al.*, 1983). Purified G_A or G_B in bicarbonate buffer (pH 9.6) was absorbed to EIA plates (Dynatech Immulon-2) at 25 ng/well overnight at 4°C . The wells were washed with phosphate-buffered saline (PBS) and then incubated for 2 h with predetermined concentrations of biotinylated MAbs and an excess of unlabelled competitor MAbs (50 μg) in PBS-0.5 M-NaCl-2% horse serum. MAb-binding avidity studies were initially performed and the concentration of unlabelled competitor ensured that epitope saturation was maximal. The plates were washed, and horseradish peroxidase (HRPO)-avidin was added for 1 h, followed by substrate. Plates were read using a Dynatech MR600 microplate reader. The degree of competition was arbitrarily defined as the following: $-$, $<25\%$; \pm , 25 to 60%; $+$, $>60\%$; \uparrow , $>100\%$ increased binding.

Animal protection experiments. Adult cotton rats (100 to 230 g) were anaesthetized with penthrane and injected intraperitoneally with MAb (2 $\mu\text{g/g}$ body weight) in groups of four (experimental) or five (control groups). One h later the animals were intranasally infected with 10^5 p.f.u. of either Long or 18357 RSV. On day 4 the animals were exsanguinated under penthrane anaesthesia, and the lungs removed under sterile conditions, weighed and homogenized in MEM-5% FCS. The homogenates were fast-frozen and stored at -70°C . Virus titrations were performed on HEP-2 monolayers in quadruplicate and the titres recorded as TCID₅₀/g lung tissue. Endpoint titres were confirmed by IFA for RSV antigen on the first negative wells.

RESULTS

A total of 19 MAbs were available: six from immunization with G_A , 11 by immunization with G_B and two by immunization with live Long RSV. All but three MAbs (L7, L9 and K6) were RSV subtype-specific by indirect immunofluorescence on infected HEP-2 cells or by EIA using

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C3
C4
C6
C7
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Table 1. Summary of IFA, EIA and plaque reduction assays

MAb Designation	Immunizing antigen route	Immunoglobulin class and subclass	IFA and EIA reactivity		Neutralization at 1:50 dilution (1:1600 dilution) (%)	
			Long	18537	Long	18537
C1	G _B (i.p.)	G2b	—	+	0	65
C3	G _B	G1	—	+	0	24
C4	G _B	G1	—	+	0	70
C6	G _B	G1	—	+	0	0
C7	G _B	G1	—	+	0	22
C8	G _B	G1	—	+	0	60 (56)
C10	G _B	G2b	—	+	0	65
C11	G _B	G2b	—	+	0	0
C12	G _B	G1	—	+	0	50
C13	G _B	G2b	—	+	0	54 (54)
C14	G _B	G2b	—	+	0	0
L7	Long RSV (i.n.)	G2a	+	+	62 (60)	87 (79)
L9	Long RSV	G2a	+	+	50 (50)	62 (73)
K1	G _A (i.p.)	G	+	—	39	0
K2	G _A	G	+	—	62 (0)	0
K5	G _A	G1	+	—	28	0
K6	G _A	G1	+	+	62 (54)	49 (54)
K8	G _A	G	+	—	58 (48)	0
K9	G _A	G	+	—	24	0

purified G in the solid phase (Table 1). Western blot analysis confirmed that L7, L9 and K6 react with both the Long and 18537 G proteins (Fig. 1a to c). In addition, K8 weakly cross-reacted with G_B by Western blot, although not by IFA or EIA.

Differing patterns of reactivity to the G proteins were evident by Western blot using infected cell lysates as the antigen. Electrophoresis of both intrinsically radiolabelled and purified G protein has revealed multiple low M_r bands ranging from 32K to 50K representing the protein backbone (G₃₂) and partially glycosylated forms of G (G₄₅₋₅₀) which electrophorese in a ladder-like pattern (Lambert, 1988; Fernie *et al.*, 1983). MAbs K1, K2, K5 and K9 reacted almost exclusively with the fully glycosylated form of G (G₉₀), while K6, K8, L7 and L9 also reacted relatively strongly with smaller forms of G. The M_r values of these bands ranged between 25K and 50K and probably represent partially glycosylated forms of G, as described above. Among the G_B-specific MAbs, C1, C4, C11 and C14 had an identical pattern and reacted more strongly with G₃₂ than with G₉₀. C6 and C8 reacted almost exclusively with G₉₀, as did K8, and the remaining MAbs reacted at least weakly with the partially glycosylated precursors as well as with G₉₀. L7 reacted strongly with many forms of G_B, similar to its reactivity with G_A.

The results of neutralization tests performed by plaque reduction assays with 1:50 dilutions of MAb without complement added, are shown in Table 1. At least 50% neutralization of Long RSV was noted for L7, L9, K2, K6 and K8. With all MAbs tested, a sizeable, persistent, non-neutralizable fraction remained even at very low dilutions of MAbs. This is distinctly different from our experience with MAbs to F protein in which nearly complete neutralization at low MAb dilutions is the rule (unpublished observation). This partial neutralization persisted at all dilutions up to 1:1600 for each of the indicated MAbs (Table 1), except K2 which neutralized only at the lowest dilution tested. K1, K5 and K9 also neutralized Long RSV, although to a much more limited extent. Some degree of neutralization of 18537 RSV was noted for C1, C3, C4, C7, C8, C10, C12 and C13 and, similar to Long RSV, consistently left a sizeable non-neutralized fraction. Most significant was that L7, L9 and K6, the three MAbs cross-reactive by EIA, IFA and Western blot, neutralized both the Long and 18537 RSV strains well.

Competitive binding studies were then performed to determine epitope specificity of the MAbs on G_A and G_B. Unfortunately, biotinylation altered binding of some of the MAbs and

(a) C12 K1 K2 K5 K6 K8 K9 L7 L9

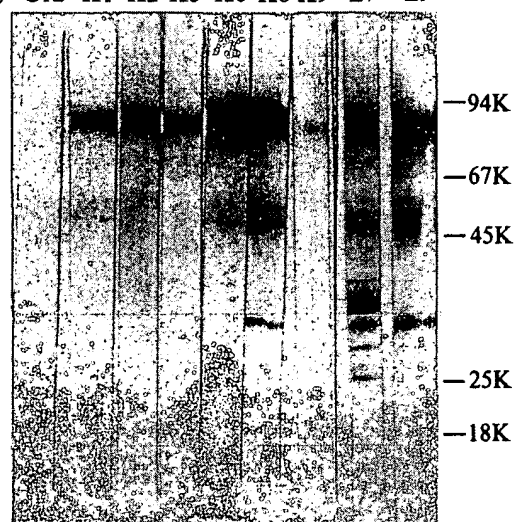
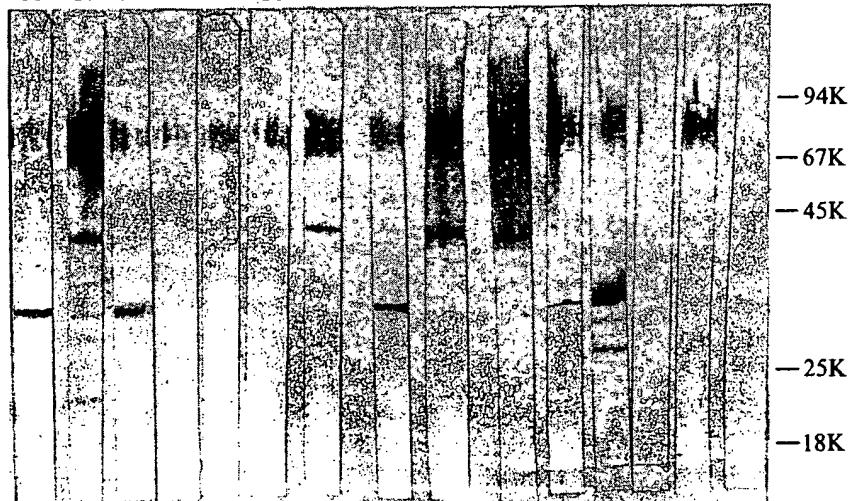
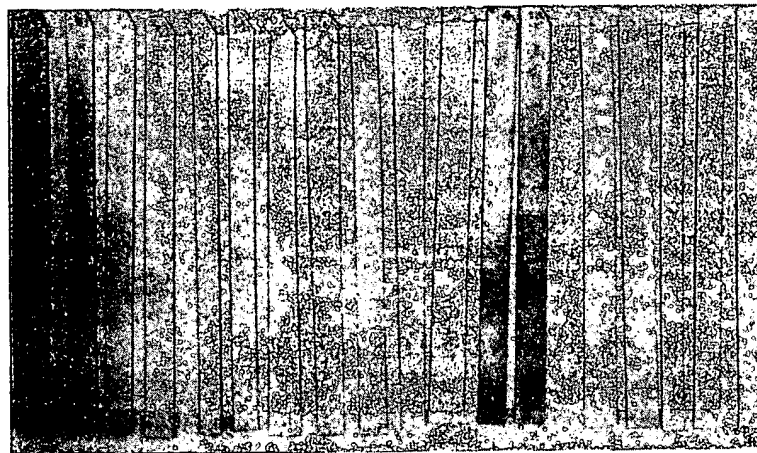


Fig. 1. MAb reactivity with RSV proteins by Western blot. Lysates of HEP-2 cells were separated by PAGE and transferred to nitrocellulose paper. The paper was cut into strips and incubated with a 1:200 dilution of MAb ascites. *M*, markers are in the right-hand lane. (a) MAb reactivity with Long (subtype A) RSV, (b) MAb reactivity with 18537 (subtype B) RSV, (c) MAb reactivity with uninfected HEP-2 cells (control).

(b) C1 C3 C4 C6 C7 C8 C10 C11 C12 C13 C14 L7 L9 K6 K8

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Table 2. *Competitive binding assays of MAbs to G_A*

Unlabelled competitor	Biotinylated MAb				
	K2	K6	K8	L7	L9
K1	—*	—	—	—	—
K2	+	—	—	—	—
K5	—	—	—	—	—
K6	—	+	+	+	+
K8	—	+	+	+	+
L7	—	+	+	+	+
L9	—	+	+	+	+
K9	—	—	—	—	—

* —, <25% competition, +, >60% competition.

Table 3. *Competitive binding assays of MAbs to G_B*

Unlabelled competitor	Biotinylated MAb							
	C3	C7	C12	C13	L7	L9	K6	K8*
C3	+†	+	+	+	↑	↑	↑	↑
C7	+	+	+	+	+	+	+	
C10	+	+	+	+	+	+	+	
C12	+	+	+	+	+	+	±	
C13	+	+	+	+	+	+	±	
C1	—	—	—	—	↑	↑	↑	↑
C4	—	↑	—	—	—	—	↑	↑
C6	—	↑	—	—	↑	↑	↑	↑
C8	—	—	—	—	—	—	↑	↑
C11	—	—	—	—	—	—	↑	
C14	—	—	—	—	—	—	—	
L7	±	±	±	+	+	+	+	
L9	±	+	±	+	+	+	+	
K6	—	+	+	+	+	+	+	
K8	—	—	—	—	—	—	—	

* K8 bound to G_B only in the presence of specific MAbs.

† —, <25% competition; ±, 25 to 60% competition; +, >60% competition; ↑, >100% increased binding.

thus, complete bidirectional competition studies could not be performed. The loss of binding activity did not correlate with the binding avidity of the native MAb. Results of competition assays with G_A are shown in Table 2. At least three non-overlapping antigenic sites on G_A were defined (designated A1, A2 and A3). Four of the MAbs neutralizing the Long strain most (L7, L9, K6, and K8) recognize a single antigenic site on G_A (A1), whereas K2 recognizes a separate site (A2). K1, K5 and K9, although not successfully biotinylated, probably bind to at least one other distinct antigenic site (A3) since they do not compete with any of the biotin-labelled MAbs.

Results of binding studies with G_B are shown in Table 3. The three MAbs that cross-react by IFA, neutralization and Western blot (L7, L9 and K6) also bind to a single site on G_B (designated site B1), similar to their reactivity with G_A. Overlapping this antigenic site is a group of four G_B-specific MAbs (C7, C10, C12 and C13) which compete bidirectionally with L7, L9 and K6 on G_B. MAb C3 is also associated with this site but, paradoxically, enhances the binding of L7, L9 and K6, and interestingly allows K8 binding to G_B. K8, which does not bind to G_B when incubated alone, also binds to G_B in the presence of several other G_B-specific MAbs (C1, C4, C6 and C8). Since K8 neutralizes subtype A RSV (Long strain) relatively well (48% at 1:1600 dilution), it was of interest to determine whether C3, a weak neutralizer of strain 18537 (24%), or C4, a better neutralizer (70%), would synergistically neutralize 18537 RSV when

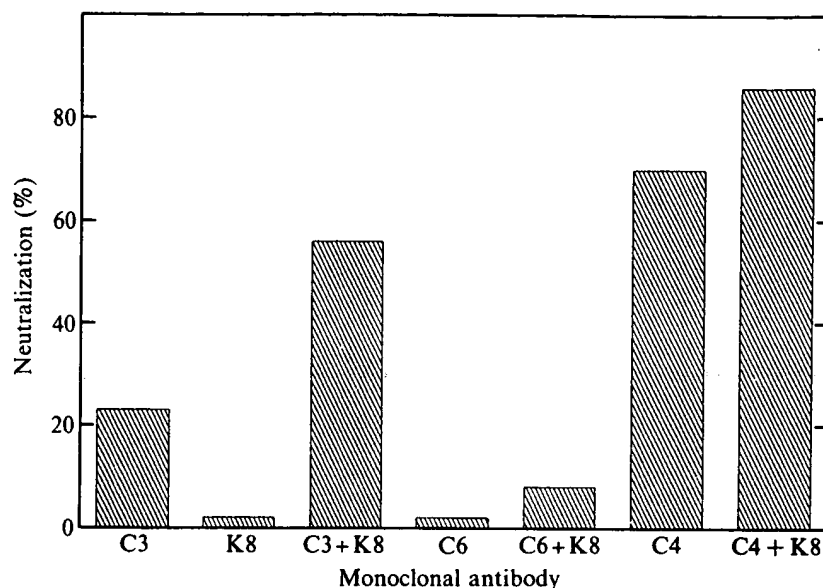


Fig. 2. Synergistic neutralization of 18537 RSV. A 1:50 dilution of each MAb was mixed with an equal dilution of an irrelevant MAb (51 is a 17D yellow fever virus MAb) or with another G-specific MAb, and incubated with 100 p.f.u. of 18537 RSV. The percentage neutralization was calculated by plaque reduction for each combination.

combined with K8. Experiments confirmed that mixtures of C3 and K8 resulted in enhanced neutralization of 18537 RSV (Fig. 2) but only slight enhancement of C4 neutralization was noted. The correlation between enhanced binding and enhanced neutralization was not always true, since minimal synergistic neutralization of 18537 RSV was noted when the non-neutralizing MAb C6 was mixed with K8, despite the fact that C6 also permitted binding of K8 to G_B in the solid-phase assay.

Unfortunately, biotinylated MAbs C1, C4, C6, C8, C11 and C14 did not bind to G_B sufficiently well to perform competition studies and thus the exact number of other antigenic sites present on G_B cannot be definitely determined. However, the Western blot patterns of C1, C4, C11 and C14 (reacting principally with G_{32}) and C6 and C8 (which react principally with G_{90}) suggest that two additional antigenic sites may be present.

In order to determine whether the cross-reactive neutralizing MAbs provided protection from challenge with both RSV subtypes, cotton rat protection studies were performed. Passive administration of L7, L9, K6 and K8 provided significant pulmonary protection from challenge with Long RSV, whereas K9 (a weak neutralizer) and K2 (a neutralizer only at high concentration) did not confer resistance (Fig. 3a). Of note, L7, L9 and K6 also provided significant pulmonary protection from 18537 RSV challenge (Fig. 3b). In addition, all of the G_B -specific MAbs tested significantly reduced 18537 RSV replication. In this case, protection was not related to neutralizing capacity *in vitro* since a non-neutralizer (C14) also reduced lung virus titres. Therefore, it may protect animals by complement-enhanced neutralization or by antibody-dependent cell-mediated cytotoxicity, as has been demonstrated in MAb-induced passive protection in mice (Taylor *et al.*, 1984). In an attempt to extend the *in vitro* synergistic neutralization by C4 and K8 to the animal model, cotton rats were passively administered C4 alone, K8 alone, and C4 plus K8 prior to challenge with 18537 RSV. Pulmonary virus titres in animals given K8 were similar to control animals ($5.8 \log_{10}$ compared to $5.6 \log_{10}$) and C4 alone markedly reduced viral titres ($3.6 \log_{10}$, $P = 0.005$). The addition of K8 to C4 significantly reduced pulmonary titres even further ($2.2 \log_{10}$, $P = 0.05$ compared to C4 alone).

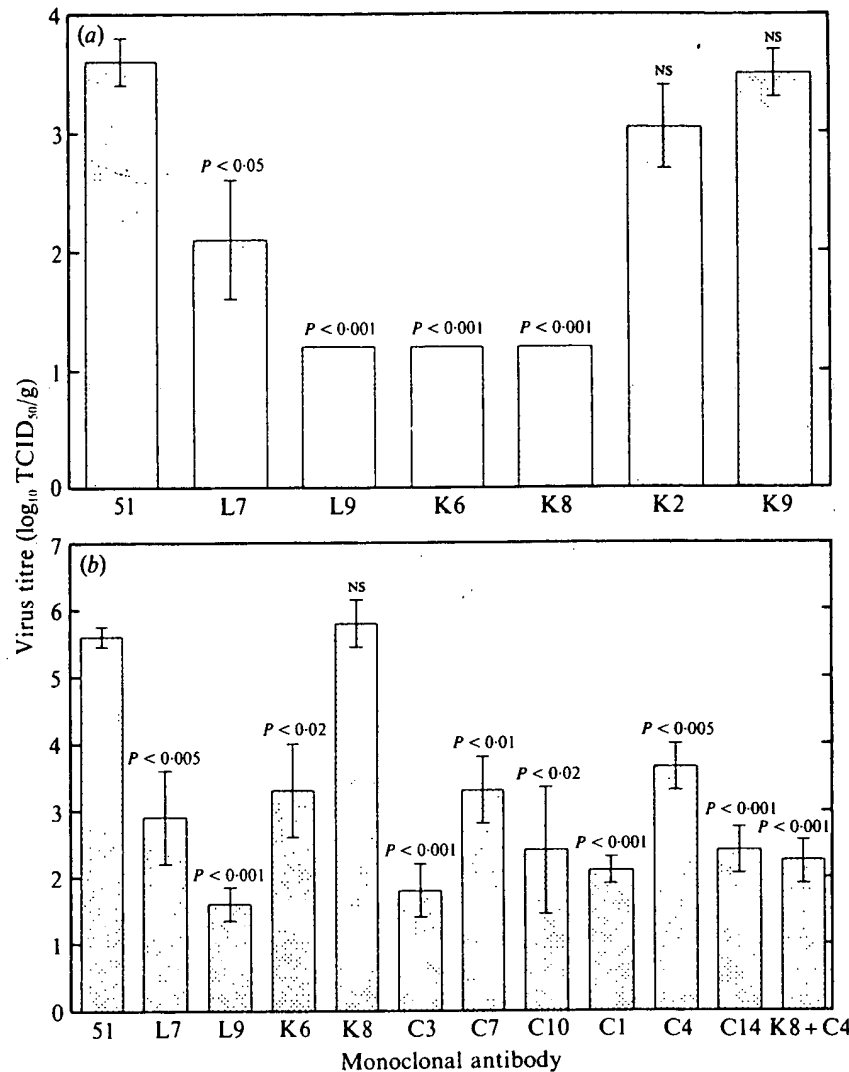


Fig. 3. Cotton rat protection from a subtype A (Long) RSV (a) and subtype B (18537) RSV (b) challenge. Groups of cotton rats were injected intraperitoneally with MAbs (2 µg/g body weight) 1 h prior to challenge with 10⁵ p.f.u. of the designated virus. MAb 51 is a 17D yellow fever envelope glycoprotein MAb. At day 4, the lungs were homogenized and viral titres were determined. Results are reported as means ± S.E. P values above each bar were calculated by Student's *t*-test; NS, not significant.

DISCUSSION

The development of an effective vaccine for RSV has been elusive, with both live-attenuated and killed vaccines proving unsuccessful. Although the role of immune mechanisms in human diseases has not been clarified, a large body of information has been developed regarding the antigenic and functional properties of RSV envelope glycoproteins. The fusion protein (F) and the attachment protein (G) have been identified as important targets of the immune system, specifically humoral immunity. It is therefore critical to understand the antigenic relationships between the envelope glycoproteins of the two major subtypes of RSV. Previous studies have shown that the F proteins of A and B subtypes are structurally and antigenically similar, and that immunization of mice with vaccinia virus recombinants expressing A strain F conferred protection from challenge with A and B subtype viruses (Stott *et al.*, 1987; Johnson *et al.*, 1987a). These results suggest that a subunit RSV vaccine containing an F protein from either subtype is likely to induce cross-protective immunity. Conversely, the structural and antigenic relatedness of the two subtype G proteins is low. We have previously shown that polyclonal monospecific

antibody to purified G_A or G_B has little cross-reactivity by EIA or Western blot and virtually no cross-neutralizing capacity. Consistent with this, Stott *et al.* (1987) and Johnson *et al.* (1987a) noted that vaccinia virus recombinants expressing G_A provided significantly less protection in both mice and cotton rats from B subtype virus challenge than from homologous virus challenge.

In the work presented here, we have explored the antigenic relationships of subtype-specific G proteins in more detail. Of 19 MAbs, all but three were subtype-specific, and at least three unique antigenic sites were present on G_A and two on G_B . Sites A1 and A2 on G_A were both neutralizing sites, and antibody binding to site A1 provided protection in the cotton rat model. MAbs defining both antigenic sites on G_B are neutralizing, although not all MAbs directed to these sites neutralized the virus *in vitro*. Importantly, however, passive administration of both non-neutralizing and neutralizing antibodies directed against these sites did provide *in vivo* protection.

The most significant finding was of a common cross-neutralizing antigenic site (A1 and B1), and MAbs to this site provided significant cross-protection. This was surprising since polyclonal antibody failed to cross-neutralize virus *in vitro* and active immunization with vaccinia virus G_A results in only marginal cross-protection (Walsh *et al.*, 1987b; Stott *et al.*, 1987; Johnson *et al.*, 1987b). The epitopes recognized by L7, L9 and K6 appear to be stable under the denaturing effects of SDS-PAGE and Western blotting and appear not to be carbohydrate-dependent since antibodies to this site (L7, L9, K6) recognize the partially glycosylated (G_{32} and G_{45-50}) forms of the G protein.

The precise locations of antigenic sites A1 and B1 are unknown. Based upon published sequence data, the longest conserved region of G_A and G_B is a 13 amino acid stretch (residues 164 to 176) in the extracellular portion of the molecule and would be a possible site for the cross-reactive epitope (Johnson *et al.*, 1987a). Approaches to determine the specific epitope include (i) sequence analysis of site A1 or B1 MAb-resistant mutants, (ii) reactivity of site A1- or B1-specific MAbs with enzymically or chemically derived fragments of G_A or G_B and (iii) reactivity of these MAbs with synthetic peptides of G_A or G_B . In a recent report, Norrby *et al.* (1987) found that sera from animals immunized with A or B subtype RSV reacted with synthetic peptides from multiple regions of G, including a peptide containing part of the conserved region. However, the only subtype cross-reactive MAb tested failed to react with any synthetic peptides.

Another characteristic of site B1 was that site A1-specific MAb binding was enhanced by several G_B -specific MAbs, suggesting that this binding site was better exposed under certain conditions. Consistent with this, SDS treatment of G_B prior to PAGE allowed K8 to recognize G_B on Western blotting, while failing to react by EIA, IFA or neutralization. The biological relevance of this was evident by the synergistic *in vitro* neutralization and *in vivo* protection against 18537 RSV by combinations of MAbs.

In conclusion, these data suggest that it may be possible to utilize the common A1/B1 antigenic site to induce a broadly protective immune response to the G protein, which when combined with the F protein may provide the optimal design of an RSV vaccine. However, further work must confirm that this cross-neutralizing, cross-protective antigenic site is present on all RSV strains, since Örvell has demonstrated the existence of antigenic diversity in the G protein of B subtype viruses and has subdivided them into B1 and B2 subgroups (Örvell *et al.*, 1987).

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